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FEED PRODUCT

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Abstract

An equine feed product comprising one or more strains of *Lactobacillus* which have been isolated from the gastric or intestinal mucosa of horse and having the ability to colonize in the equine intestines. The invention especially refers to the new strains *Lactobacillus plantarum* JI:1, which has been deposited under the number DSM 11520, and *Lactobacillus* species AC:3, which has been deposited under the number DSM 12429, an oatmeal gruel fermented with said strains, and use thereof for the prophylaxis or treatment of disturbances in the equine intestinal microflora, as well as gastritis.

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(21) International Application Number: PCT/SE98/01796 (22) International Filing Date: 5 October 1998 (05.10.98) (30) Priority Data: 9703606-5 3 October 1997 (03.10.97) SE (71) Applicant (for all designated States except US): PROBI AB [SE/SE]; Forskningsbyn Ideon, S-223 70 Lund (SE). (72) Inventors; and (75) Inventors/Applicants (for US only): JOHANSSON, Marie-Louise [SE/SE]; Flygelvägen 14, S-224 72 Lund (SE). LÖNNER, Clas [SE/SE]; Rolsberga 4, S-241 94 Eslöv (SE). (74) Agents: LARFELDT, Helene et al.; Bergenstråhle & Lindvall AB, P.O. Box 17704, S-118 93 Stockholm (SE).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CI, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>With an indication in relation to deposited biological</i> <i>material furnished under Rule 13bis separately from the</i> <i>description.</i>
(54) Title: FEED PRODUCT (57) Abstract <p>An equine feed product comprising one or more strains of <i>Lactobacillus</i> which have been isolated from the gastric or intestinal mucosa of horse and having the ability to colonize in the equine intestines. The invention especially refers to the new strains <i>Lactobacillus planiarum</i> J1:1, which has been deposited under the number DSM 11520, and <i>Lactobacillus</i> species AC:3, which has been deposited under the number DSM 12429, an oatmeal gruel fermented with said strains, and use thereof for the prophylaxis or treatment of disturbances in the equine intestinal microflora, as well as gastritis.</p>		

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FEED PRODUCT

The present invention refers to new strains of the genus *Lactobacillus*, and an equine feed product comprising one or
5 more of said strains.

BACKGROUND OF THE INVENTION

Disturbances in the equine intestinal flora can bring about a deteriorated ability to take up the nutriment from the
10 feed, a reduced efficiency, a deteriorated resistance to infections and accompanying infections of different kinds. Other symptoms are a changed consistency of the excrements, which are often malodorous, and an ugly coat of hair. It is well known that disturbances of the intestinal flora above all
15 appear in stress situations, for instance during long transportations or change of stable environment, too tough training and change of feed, but also in treatment with antibiotics. Problems of a disturbed intestinal flora also occur in foals, which during their first living have to obtain
20 an intestinal microflora of their own.

Different disorders which can be associated with a change of the intestinal bacterial flora are inflammations in the intestinal mucosa and gastric ulcer, and bacterial infections giving for instance acute colitis, such as intestinal
25 chlostridiosis, manifesting in diarrhea and toxemia and caused by i.a. *Clostridium difficile*, and colic, such as Baron-Gruff-disease, which is caused by *Clostridium perfringens*.

The presence of coliform bacteria in the excrements has since long been used to judge the state of health in horses as
30 to disturbances of the intestinal flora. A high content of said bacteria has been considered to indicate that the intestines are in good condition, while a low content thereof, as well as a high content of *Bacillus* and *Clostridium* spp, respectively, have been associated with intestinal disturbances. The presence
35 of moulds and other fungus also have been considered to have an injurious effect on the intestinal flora. In addition a reduced body temperature, lower than 37°C, has been considered to indicate problems in the gastrointestinal tract. The normal temperature in horses is within the range 37.3 - 37.8°C.

Lactobacilli is one of the main groups of microorganisms in the intestinal microflora of healthy horses. Fujisawa et al. discloses in Bifidobacteria Microflora, Vol. 12(2), p. 87-90, 1993, the isolation of 286 strains of lactobacilli from the gastrointestinal tract of healthy horses. The main part of the strains could be identified as belonging to the species *L. salivarius*, *L. acidophilus* and *L. reuteri* and the last two species were found in all parts of the gastrointestinal tract while *L. salivarius* was not isolated from the stomach. It is also important to remember that the microflora is also influenced by the type of feed that the horse eats.

Probiotics, that is different bacterial and yeast preparations which are orally administrated with the purpose to improve the health in man and animals, have since long been used against intestinal disturbances to improve the intestinal function and the general state of health. As probiotics are commonly used different strains of lactic acid producing bacteria, such as strains of *Lactobacillus* and *Streptococcus*. The probiotic hypothesis says that if said bacteria can be added to the intestines in a sufficient amount during conditions of stress or illness, that is when the intestinal flora is disturbed in such a way that pathogenic bacteria are favoured, an establishment of the not wanted bacterial strains in the intestines will be counteracted. To maintain a healthy intestinal flora would then be decisive to prevent those disorders which are associated with a disturbance of the intestinal flora.

In Fox, Veterinary Medicine, August 1988, Probiotics: Intestinal inoculants for production animals, a review is made of different commercial probiotics used in raising different animals, especially chickens, pigs and calves.

PRIOR ART

It is today common to feed horses having malodorous excrements with sour milk or yoghurt to overcome the underlying disturbances in the stomach and intestines. Equine products on the market are for instance Bakteriebalans (Lantmännen, Sweden) and Lactosat®häst (Ewos Sverige AB, Södertälje) which both contain three different strains of lactic acid producing

bacteria, that is of *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *Streptococcus faecium*. Said agents have in common that the lactobacilli which are present in the administrated products either are such which are naturally prevailing in the fermented product or such which have been isolated from man.

Another agent which has been used against equine intestinal disturbances is ColiCure (VETTECH AB, Stockholm), a product consisting of a purified and stabilized strain of *Escherichia coli*, which was originally isolated from horse, in a concentration of $>10^9$ viable bacteria/ml. The same principle is behind the administration of "mash", that is dispersed excrements from a healthy horse, that is to get a bacterial strain which fits into the intestinal environment and can become established therein. It is, however, controversial to add bacteria of the family *Enterobacteriaceae*, for instance *E. coli*, in view of pathogenicity and occurrence of endotoxines.

EP 0 203 586 discloses a composition for treatment of gastrointestinal disorders in animals, especially those being caused by enterotoxigenic strains of *Escherichia coli*, which is based on the strain *Lactobacillus fermentum* ATCC 53113, which has been isolated from pig. An advantage of the new composition is said to be that it is not pathogenic and acts by preventing infectious organisms from binding to the intestinal mucosa. An overview is also given of different bacteria which have been used to prevent or to remedy gastro-intestinal disorders, but which have all turned out to be effective only in part.

It has previously been shown that certain strains of *Lactobacillus plantarum* have the ability for a certain period of time to become established in the intestinal system of man. In EP 92916294.9 certain strains of *Lactobacillus*, especially *L. plantarum* 299 DSM 6595 and variants thereof having the ability to become established in the intestines and to remain for at least 11 days after oral administration, are described. Said strains have turned out to have a favourable effect on different disturbances of the intestinal microflora in man.

The normal lactobacilli flora in the intestines of different animal species are different and although the same species of *Lactobacillus* can be found in two different animal

species, the bacteria do not normally belong to the same strain of said species. The strains present differences between themselves which are of importance for their chances of becoming established in the actual environment. In order to be able to treat gastrointestinal disorders in horses, there is therefore a continuous need of a safe, non-toxic product comprising one or more bacterial strains having the ability to become established in the equine intestines.

10 DESCRIPTION OF THE INVENTION

The invention refers to an equine feed product preventing and rectifying microbially caused intestinal disturbances, which contains one or more strains of *Lactobacillus* which have been isolated from the gastric or intestinal mucosa of horse and which have the ability to colonize in the equine intestines.

Horse refers in this connection to subjects of the genus Equidae, that is in addition to horse also donkey and zebra, having a similar composition of the intestinal flora.

20 The feed product according to the invention is preferably a feed which has been fermented with a strain of *Lactobacillus* which has been isolated from the gastric or intestinal mucosa of horse and which has the ability to colonize in the equine intestines.

25 The feed product according to the invention is preferably based on a cereal based grain or supplementary feed, green forage or hay.

A preferred aspect of the invention is a feed product based on oats which has been fermented with one or more strains of lactobacilli isolated from horse, especially with any of the new strains stated below.

30 According to another aspect of the invention the feed product is an ensilage based on green forage or hay which has been fermented with one or more strains of lactobacilli isolated from horse.

35 The invention also refers to the new strain *Lactobacillus plantarum* JI:1, which has been deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH on April 21, 1997, under number DSM 11520 and variants thereof; especially

for use for prophylaxis or treatment of disturbances in the intestinal microflora.

The invention also refers to another new strain *Lactobacillus* species AC:3 and variants thereof, which has been deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH on October 2, 1998 under number DSM 12429.

The above strains have turned out to grow well on oats and give a tasty feed product, survive the passage through the gastrointestinal tract of the horse and have the ability to become established in the intestines of horse. Tests have shown that the new strain *Lactobacillus plantarum* JI:1 reduces the content of undesired bacteria in the equine intestines, for instance the total count of *Bacillus* and the total count of *Clostridium* and seems to improve the immune response of the horse.

The feed product according to the invention can be a supplementary feed or a feed, in solid or liquid form. The feed product can be a grain or supplementary feed based on any type of feed which can be fermented by the selected strains of lactobacilli, for instance cereals, such as oats, i.e. black oats, barley, wheat, rye, sorghum or corn, or vegetables, such as leguminous plants, especially soybeans, root vegetables and cabbage, or green forage, such as grass or hay.

Oats is a preferred feed owing to the high content of vitamins and minerals, the well-balanced amino acid composition, the contents of polar lipids and fibres. A variety of oats having a higher fat content has recently turned out to be an appropriate feed for high capacity horses.

A preferred aspect of a feed product according to the invention can be prepared from oats in accordance with the international patent application W089/08405 referring to the preparation of a nutrient composition. Other examples of preparation of preferred feed products are given below.

The invention also refers to the use of one or more strains of *Lactobacillus* which have been isolated from the gastric or intestinal mucosa of horse having the ability to colonize in the equine intestines.

The invention also especially refers to the use of the new strain *Lactobacillus plantarum* JI:1 or a variant thereof

for the prophylaxis or treatment of disturbances in the equine intestinal flora. Also inflammatory conditions in the gastric or intestinal mucosa can be alleviated. The invention especially refers to the use of said strain for the preparation of a product for prophylaxis or treatment of disturbances in the equine intestinal flora.

A feed-product according to the invention can be administered before or in connection with stress situations, for instance during the warming up before a race, transportation etc. to horses which on occasions like those are stricken by diarrhea; to improve the common condition of horses which are thin, have a poor appetite or a bad coat; to make horses less sensitive to infections, that is to strengthen the immune response; during treatment with anti-biotics to prevent the disturbances of the intestinal flora which normally appear, and thus the diarrhea which is often associated with treatment with antibiotics; to horses having a gastric ulcer; to horses having a diarrhea or malodorous excrements which might be infected with *Clostridium difficile* or any other pathogenic microorganism.

The content of lactobacilli in the feed should not be below $5 \cdot 10^8$ CFU/g or ml and an adequate dose to grown up horses is $2 \cdot 10^{12}$ CFU per day, which corresponds to about 2-4 litres of the oatmeal gruel exemplified below or about 100-200 ml of a freeze culture as below. Higher contents can without any risk be used on special occasions. When administrated to fowls a smaller amount should be given.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows a photography of an electrophoretic gel with strains which have been analysed by RAPD.

Figure 2 shows a photography of an electrophoretic gel with strains which have been analysed by REA.

EXPERIMENTAL PART

In order to produce the new strain representative strains of *Lactobacillus* have firstly been isolated from horse, said strains have then been classified and examined as to ability to ferment oats and rapidly give an adequate pH-reduction, good

taste and smell, and finally feed experiments on horses have been done to evaluate the effect obtained.

Isolation of strains of lactobacilli

5 The lactobacilli flora in the gastrointestinal tract of
10 healthy, freshly slaughtered horses were investigated by
taking biopsies of 1-2 cm² of the intestinal mucosa from three
different places, that is the lower stomach (pylorus), the
small intestine (ileum) and the large intestine (colon). The
10 specimens were rinsed directly and carefully with sterile
transport solution (0.9 g NaCl, 0.1 g peptone, 0.1 g Tween 80,
0.02 g cysteine per litre), in order to eliminate contamination
from bacteria in the excrements, and were kept cool during the
transportation to the laboratory. There the specimens were
15 treated in an ultrasonic bath for 5 minutes and then in a tube
shaker for 2 minutes, in order to loosen the bacteria from the
intestinal mucosa. In total 51 samples were taken.

Identification of the lactobacilli flora in the gastrointestinal tract in horse

20 From each sample 1 or 2 lactobacilli isolates were taken
and characterized as to ability to ferment 49 different carbon
sources according to the API 50 CH system (API Systems S.A.,
Montalieu, Vercieu, France). This normally gives species
specific patterns and the results obtained were compared to the
25 results of about 200 different, known strains of *Lactobacillus*
comprising all type strains of all acknowledged species of
lactobacilli. The following result was obtained:

	<u>Species</u>	<u>% of the isolates</u>
	<i>L. salivarius</i>	14
30	<i>L. salivarius</i> -like	29
	<i>L. reuteri</i> -like	14
	<i>L. johnsonii</i>	4
	<i>L. johnsonii</i> -like	10
	<i>L. plantarum</i>	12
35	<i>L. animalis</i>	6
	<i>L. brevis</i>	2
	<i>L. vaccinostercus</i> -like	2
	<i>L. murinus</i>	2
	<i>L. species</i>	4

Selected strains and characterizations

The isolated bacterial isolates were then investigated as to ability to ferment oatmeal gruel, which had been prepared as follows.

- 5 18.5% by weight oatmeal (MP-450, Kungsörnen AB, Järna)
0.925 % by weight malted barley meal (MBF, Nord Malt AB,
Söderhamn) were mixed with tap water and heated to 95°C while
slowly stirred. After 30 minutes the gruel was cooled to 37°C
and fermented with the different strains for 20 h to a pH below
10 4. Especially the rate of reduction of the pH and the smell and
aroma of the final product were investigated. The best results
were obtained when using the isolate *L. plantarum*
and *L. salivarius*-like as a starter culture and the worst
results were obtained with *L. reuteri* and *L. johnsonii*-like.

- 15 Two strains were selected based on the above. The strains
were identified phenotypically on API 50 CH and genotypically
by means of the analysis methods RAPD and REA as *Lactobacillus*
plantarum JI:1 and *Lactobacillus* species AC:3, respectively. In
this process purified isolates were cultured over night in
20 broth. Before inoculation in API 50 CHL medium the cultures
were washed once in 0.9 % NaCl-solution. The samples were read
after 2 d and 7 d. The results were evaluated from 0 (violet)
to 5 (yellow) and a value of 3-5 was regarded as positive.

25 Genotype characterization

Randomly amplified polymorphic DNA (RAPD)

- RAPD is a polymerase chain reaction (PCR) consisting of
repeated cycles of template denaturation, primer annealing and
primer extension. This will give an amplification of certain
30 fragments of the genome.

- Purified bacterial isolates were cultured over night in
Lactobacillus Carrying Medium (LMC, Efthymiou and Hansen, 1992)
with 1 % glucose. Cells from 100 µl of the culture were
recovered by centrifugation and washed twice in 1 ml sterile
35 distilled water. The cells were resuspended in 0.25 ml sterile
distilled water and then disintegrated by vigorous shaking with
glass beads (diameter 0.2 mm) using an IKA Vibrax VXR shaker
(IKA Labortechnik Staufen, Germany) at the maximum speed for 30
minutes in the cold. The cell debris were pelleted by

centrifugation and 1 μ l of the supernatant was used for PCR. PCR was performed in a DNA Thermal Cycler 480 (Perkin Elmer, Norwalk, US) using a 9-mer primer (ACGCGCCCT; Pharmacia Biotech, Sweden) in a concentration of 8 μ mol/l. The
5 nucleotides were added at a concentration of 0.2 mmol/l of each dNTP (Perkin Elmer; Branchburg, NJ, US). The Taq-polymerase concentration was 50 U/ml (Boehringer Mannheim) and the buffer concentration was 100 U/ml (10 x PCR buffer; Boehringer Mannheim). The reaction mixture was covered with mineral oil
10 and subjected to the following temperature cycles: 94°C, 45 s; 30°C, 120 s; 72°C, 60 s during four cycles and then to 94°C, 45 s; 36°C, 30 s; 72°C, 30 s for 26 cycles (the extension step is made 1 s longer for each new cycle). The PCR process was completed at 75°C for 10 min and the sample was
15 then cooled to 4°C.

Gel electrophoresis was performed by adding 20 μ l of the sample to a submerge, horizontal agarose gel plate consisting of 75 ml 1.5% agarose (type III: High EEO, Sigma, St. Louis, US) of a size of 150 x 115 mm. Gels were run for about 2.5 hours at 100
20 V in TB electrophoretic buffer (89 mM boric acid, 89 mM Tris-OH, 2.5 mM EDTA, pH 8.3) without cooling. 0.5 μ mol of a DNA molecular weight marker VI (Boehringer Mannheim Scandinavia, Bromma, Sweden), was used as a standard. After electrophoresis the gels were stained in ethidium bromide (3 μ g ml⁻¹) for 5
25 minutes and then washed for 10 minutes and made visible at 302 nm with a UV transilluminator (UVP Inc., San Gabriel, California, US) and photographed.

In Figure 1 the RAPD-pattern of, from the left, *L. plantarum* JI:1, the type strain of *L. plantarum* ATCC 149 17^T
30 and the marker are shown. From this can be seen that the pattern of this new strain according to the invention is slightly different from the pattern of the type strain. In the same way the proposed strain *Lactobacillus salivarius*-like AC:3 (according to API) differed from the type strain of
35 *L. salivarius* ssp. *salivarium* ATCC 11741 (data not shown).

Purification of chromosomal DNA

Preparation and purification of chromosomal DNA were performed according to the method described by Ståhl et al.,

International Journal of Systematic Bacteriology 40:189-193, 1990. The strain were cultivated over night at 37°C in 100 ml broth with 1 % glucose and were then harvested and washed in TES (50 mM NaCl, 100 mM Tris, 70 mM sodium EDTA, pH 8.0) and
5 suspended in 3 ml TES + 25 % (weight/volume) sucrose. 2 ml TES + 25% sucrose supplemented with 120 mg lysozyme (quality VI, Sigma) and 140 U mutanolysine (Sigma) were added. The cells were incubated at 42°C for 2 h; 3 mg proteinase K (quality XI, Sigma) were then added and the suspension was further incubated
10 at 37°C for 30 minutes. After cooling on ice 1 ml of a 5 % NaCl solution was added, 1 ml 20 % dodecyl sulphate and 11 ml TES and the suspension was carefully stirred with a glass bar and heated to 65°C. The cell lysate was then extracted once with redistilled phenol-chloroform (1:1) and once with isoamyl
15 alcohol-chloroform (1:24). DNA was then precipitated with ethanol at 20°C.

The chromosomal DNA was then purified and separated from the covalently bound closed circular plasmid DNA by density centrifugation in a CsCl-gradient with ethidium bromide
20 according to Bauer and Vinograd, Journal of Molecular Biology 33, 141-171, 1968. The DNA concentrations were determined in a fluorometer (model TKO 100 Hoefer Scientific Instruments, San Francisco) with the dye Hoechst 33258 according to the
recommendations of the manufacturer.

25 *Restriction endonuclease analysis (REA)*

Portions of 0.75 µg purified, chromosomal DNA were digested separately at 37°C for 4 h with 10 U *Hind*III, *Cla*I and *Eco*RI (Boehringer Mannheim). Gel electrophoresis was performed
30 by using submerge horizontal agarose gel plates consisting of 150 ml 0.9 % agarose (ultrapure DNA quality, BioRad Laboratories) and they were mulded as gel plates. 0.2 µg of a high molecular weight DNA marker (BRL) together with 0.5 µg of a DNA molecular weight marker VI (Boehringer Mannheim) were
35 used as standards. In addition pHC 79, digested with *Eco*RI, was added to each well as an internal standard. Minimal band distorsion and maximal sharpness was reached by adding the DNA sample in a Ficoll loading buffer (2 g Ficoll, 8 ml water, 0.25 % bromophenol).

The fragments were separated on 0.9 % agarose gels at 8.5°C and 40 V for 18 h and were then stained for 20 min in ethidium bromide (2 µg/ml) and destained in distilled water and photographed. By this a well dispersed and fairly well separated bands were obtained down to a molecular weight of 1.2×10^6 , see Figure 2. This figure shows the REA pattern of the *Lactobacillus plantarum* strains JI:1, 299v and ATCC 149 17^T (type strain) after sequential treatment with the restriction enzymes *Ecco* R1, *Cla* 1 and *Hind* III, respectively. The first and the last lane shows a marker. The strain AC:3 was analysed by REA and found to be rather different from the type strain of *Lactobacillus salivarius* ssp. *salivarius* (data not shown).

Examples of feed compositions

15 Preparation of oatmeal gruel in industrial scale

Oatmeal (318 kg), malted barley flour (12.8 kg) and water (1682 l) are mixed and heated to 88°C, the temperature being first maintained at 55°C for 1 h and then at 88°C for 0.5 h. The mixture was then cooled to 38°C which requires about 0.5 h. At this temperature the starter culture *Lactobacillus plantarum* JI:1 is inoculated in an amount of 2 l to 2000 l of the mixture, giving a concentration of $2 \cdot 10^7$ CFU/ml. The whole is incubated at 38°C for 15-20 h, after which pH should have been decreased to <3.9. The fermented mixture was then cooled to 6-8°C and is after packaging and cool storage ready for distribution and sale.

The freshly prepared, final product contains $1-2 \cdot 10^9$ CFU/ml and after storage for 1-2 months the bacterial count has been reduced to about $5 \cdot 10^8$ CFU/ml.

30 The nutritional value of the above feed product is evident from the following table

<u>Nutrition value</u>	<u>per 100 g</u>	<u>per 2 kg</u>
Energy value	240 kJ = 57 kcal	4780 kJ = 1143 kcal
Protein	2.1 g	41.5 g
35 Dietary fibres*	1.5 g	29 g
Fat**	1.1 g	23 g
Carbohydrates	10 g	190 g
Vitamine B1 (thiamine)	0.07 g	1.3 g

- * according to ASP et al., J. Agric. Food Chem. 31:476-482
** according to SBR, Nordisk Metodikkommitté för livsmedel No. 131, 1989

- 5 By adding CaCO_3 in the above methods of preparation a feed composition having a higher bacterial count is obtained after a somewhat extended fermenting process (20-22 h).

By using *Lactobacillus* species AC:3 as a starter culture a corresponding oatmeal gruel is obtained.

10

Fermented carrots

To prepare a batch of fermented carrots carrots (10 kg) are roughly grated or finely divided in any other way. Water (5 l) is boiled and then allowed to cool to 40°C , when

- 15 *Lactobacillus plantarum* JI:1 (inoculant $2 \cdot 10^{11}$ CFU, corresponding to about 2 g freeze dried product or 20 ml freeze culture) and salt (100 g) are added. The grated carrots are layered with the inoculant fluid in a sterile container and densely packed. It is important that the vegetables are below
20 the surface of liquid. The container is closed and then incubated at 24°C for about 2 days. Ripening takes place in a refrigerator or cool larder for about 3-4 weeks. The fermented product can then be cool stored for about 1 year.

25

Freeze dried product

- 1 l water
- 200 g skim milk powder
- 2 g yeast extract
- 1 l bacterial suspension ($5 \cdot 10$ CFU/ml)

- 30 The above components are mixed and transferred to sterile plates which are placed on shelves in a freeze drier. The product is frozen to about -40°C before the drying is started. After about 3 d the drying is finished and the product obtained is milled to a powder which is packed into sterile bags under a
35 controlled nitrogen atmosphere. The keeping quality of the obtained product is about 2 years at freeze storage.

Freeze culture

- 1 l of a bacterial suspension having a content of $5 \cdot 10^{10}$

CFU/ml is added to 1 l of a freeze medium having the following composition.

	potassium dihydrogenephosphate	0.36 g
	dipotassium hydrogenephosphate	1.64 g
5	trisodium citrate	1.18 g
	magnesium sulphate	0.5 g
	glycerol	240 g
	distilled water	760 ml

The product obtained is frozen at -80°C in 200 ml bottles.

10

Preparation of ensilage

Ensilation is based on two principles, that is to lower the pH value of the green mass to 4.2 or lower and to exclude the air oxygen. 50 g of the freeze dried culture of
15 *Lactobacillus plantarum* JI:1 is mixed with some litres of water to solve the powder and then additional water is added and a few kg of melasses to a total volume of 50 l. When the green mass is rolled into bales and enclosed into plastics then bacterial solution is dosed into the mass in an amount of about
20 1 l per 200 kg green mass by means of dosing pump mounted on the chaff cutter or the press. It is important to get a uniform admixture of the bacterial solution so that the added bacteria can grow in the whole of the bales. When ensilage is prepared in a silo the bacterial solution is added by means of a dosage
25 pump through the fan of the silo.

Tests in horses in vivo

Administration of *L. plantarum* JI:1 in oatmeal gruel

This study comprises 10 trotters, five of which were
30 given 2 l fermented oatmeal gruel each day for 8 weeks, while the other five horses which were not given oatmeal gruel took part in the study as reference horses. Samples of excrements were taken at six different times, directly before the horses were fed with the oatmeal gruel and then after 1, 3, 6 and 8
35 weeks and finally one week after that the horses had ceased to eat the oatmeal gruel. The microbiological analyses comprised the total number of lactobacilli, *Enerobacteriaceae* and *Bacillus*. The pH value of the excremental samples were also analysed. In addition the number of *L. plantarum* JI:1 of the

total lactobacilli flora was analyzed.

In addition to the microbiological analysis also some subjective assessments of the status of the horses were done during the test period. This included consistency of the excrements, odour of the excrements, the fat of the horses, the gloss of the coat and finally the general condition of the horses.

The results show that the content of lactobacilli was substantially increased in two of the horses during the feed period. Both said horses had a low content of lactobacilli at the start of the test. From all horses which obtained the fermented oatmeal gruel the bacterium *L. plantarum* JI:1 was isolated from the excrements. The number varied from 1 to 72 % of the total lactobacilli flora. Three of four horses had *L. plantarum* JI:1 in their excrements still one week after the completion of the feeding with the oatmeal gruel.

The number of *Bacillus* spp was decreased significantly in four of the horses during the feed period and one week after, while no change could be observed as to the number of *Enterobacteriaceae* or the pH values of the excrements during or after the test.

The fermented oatmeal gruel was much appreciated by the horses and became almost as "tit bit". There were also some signs indicating that the appetite of the horses was increased.

One of the horses, Luradej, registration number 90-4843, is noted for recurrent problems with the stomach and the intestines. Concurrently with the beginning of said problems the body temperature declines to about 36°C. After taking the fermented oatmeal gruel containing the bacteria *Lactobacillus plantarum* JI:1 for two weeks the temperature rose to a normal level of 37.5°C for the first time in several months. When the horse ceased to eat the oatmeal gruel after having had it for 8 weeks the temperature again after 2-3 days decreased to about 36°C. After a short brake the horse was again fed with the oatmeal gruel which resulted in a temperature raise to the normal level. This was repeated once more at a later occasion. After two years daily intake of the described oatmeal gruel Luradej was given a bacterial concentrate of *L. species* AC:3 in the same amount. This resulted in an additional positive

response in the general condition of the horse.

During the test period one of the horses was treated with a broad-spectrum antibiotic (Gentaject vet., 1.2 g twice a day for 7 days). When a horse is treated with this antibiotic there are normally some side effects, for instance problems with the stomach and the intestines for a long time. In this case the horse received the fermented oatmeal gruel during the whole antibiotic treatment and in addition during a number of weeks afterwards and the horse had no problems whatsoever neither with stomach nor intestines. This was repeated as a later occasion with another horse and another antibiotic giving the same positive results.

Colonizing of *L. plantarum* JI:1 and the effect thereof on the content of *Enterobacteriaceae*, *Bacillus*, *Clostridium* in horse excrements

The same product which has been described in the above test was used also in this study. In all 34 horses were included in the study. Twenty-six of them were located with six different trot trainers in the south of Sweden. The horses had different backgrounds, some of the horses had a problem with loose, malodorous excrements and/or poor fat and poor gloss of the hair coat. The horses were fed somewhat different as to, for instance, type of grain and certain supplementary feeds. Some of the horses also were periodically grazing.

The horses were fed with 2 l of fermented oatmeal feed per day for two months and in certain cases for three months. A small number of horses were periodically fed with only 1 l per day. This was for instance the case when some of the horses were grazing and then not so interested in eating anything else than grass. Samples of the excrements were taken directly before the horses were fed with the fermented feed and after having had said feed for 2-3 months. The microbiological analysis comprised the total number of lactobacilli, the number of *L. plantarum* JI:1 of the total number of lactobacilli, the total number of *Enterobacteriaceae*, *Bacillus* and *Clostridium* as well as the number of *B. cereus*.

In addition to the microbiological analysis some subjective assessments of the status of the horses were also

done during the test period. This included consistency of the excrements, the odour of the excrements, fat, gloss of the coat, activity and appetite.

The results showed that the number of lactobacilli was significantly increased in the horses also in this study (p<0.05 after analysis by Wilcoxon signed ranks test). The median value before the start of the feeding was $1 \cdot 10^6$ cfu/g of excrements ($1 \cdot 10^5 - 1.7 \cdot 10^7$) and after 2-3 months of treatment $3.5 \cdot 10^6$ cfu/g ($1 \cdot 10^5 - 3.8 \cdot 10^7$). Preliminary *L. plantarum* JI:1 was found in the excrements from all horses after being fed.

The decrease in total number of *Bacillus* is very obvious (p<0.001 after analysis by Wilcoxon signed ranks test), with 80 % of the analysed horses showing decreases in these counts after intake. Also sulphite-reducing clostridial counts were significantly decreased after treatment (p<0.05 after analysis by Wilcoxon signed ranks test). Here 74 % of the analysed horses had decreased amounts of clostridia (horses showing very low counts, less than $2 \cdot 10^2$ cfu/g of excrements, were omitted from the statistical analysis). As to the number of *Enterobacteriaceae* no tendency whatsoever could be observed.

Additional observations of interest which were made during this test were as follows:

- Some of the horses had, at the start of the test, problems with diarrhea in stress situations and when warming up before a race. After having taken the fermented feed for a short time this problem ceased in all cases.
- About 10 horses had problems with loose excrements at the beginning of the test. All horses obtained after some weeks at most, normal excrements.
- Scarcely 10 horses had a problem with poor fat at the beginning of the project. All except one have after being fed with the fermented feed got a better fat.
- About 5 horses had a poor gloss of the coat at the start of the test. They all got a finer coat after having taken the fermented feed for a couple of weeks.
- Three horses have during the test period obtained antibiotics (two of the horses have obtained broad-spectrum antibiotics and one a sulpha preparation). Not in any case has the antibiotic had an adverse effect on the horses but the

fermented oatmeal feed instead seems to have had a positive effect on the horses and compensated for the side effects which are otherwise common.

- Two of the trainers had before the start of the test given sour milk to some of the horses without any noticeable result. Both were of the opinion that the effect was much better with the fermented oatmeal feed.
 - Three of the trainers had before the start of the test tested the product ColiCure on some of the horses with a poor result.
- 10 All were of the opinion that the fermented feed gave a better result.

In a summing up of the effect of the fermented oatmeal feed on the 26 horses it has in 19 cases been observed a more or less positive effect on the intestinal flora of the horses and/or the common status of the horses. Only in 7 cases no evident positive effect has been observed. In a number of said cases there are explanations, for instance eating of concentrated feed of a bad hygienic quality or other problems which have for instance brought about medication of the horse.

20

Jaffa Qui, example of horse in vivo

Jaffa Qui, registration number 92-1858, a very promising trotter, won her first race as an early three-year-old. When changing stable she was as a three-year-old for 6 months given water containing a considerable number of coli bacteria. When she was later moved to a new trainer it was gradually found that the whole stable had Rhino virus and Herpes virus. Jaffa Qui became the most ill of all the horses, probably owing to the reduced resistance caused by the bad water quality. She also got nettle rash and looked more miserable for every day. She did not cast her winter coat, became fluffy and almost stopped eating, and had no energy to training. After administration of the oatmeal gruel according to the invention, which she accepted at once and started to eat, she became better and better and it can now be declared that she has never before been so fine. She has a better fat than before and a glossing coat. She has also accepted training very well during the last months and has now qualified and made her debute.

Administration of *Lactobacillus plantarum* 299v to horses with gastritis

5 Five horses with gastritis problems, one of which had a partly cured gastritis after administration of Losec (Astra AB, Södertälje, Sweden), were treated with the oatmeal gruel described, for a number of weeks. All the horses showed full clinical recovery from the gastritis and two of the horses were 100 % recovered by gastroscopy. In one case the horse was simultaneously treated with Losec. The details, as well as the results are given in the following Table.

10

Table

Horse	Diagnosis before treatment	Dosage and duration of the treatment with <i>L. plantarum</i> JI:1	Response to the treatment	Comments
Colombine	Gastritis	$2 \cdot 10^{12}$ cfu/d for 3 months	Full clinical recovery, 100% recovery by gastroscopy	No other medication
Fantasy Pride	Gastritis	$2 \cdot 10^{12}$ cfu/d for 1,5 months	Full clinical recovery	No other medication
Faxa	Gastritis	$2 \cdot 10^{12}$ cfu/d for 1 month	Full clinical recovery	No other medication
Bali Pet	Gastritis	$2 \cdot 10^{12}$ cfu/d for 1 month	Full clinical recovery	12 d treatment with Losec
Polk	Partly cured gastritis (60-70% recovery)	$2 \cdot 10^{12}$ cfu/d for 1 month	Full clinical recovery, 100% recovery by gastroscopy	No other medication

The results show that the feed product of the invention has a potential of curing gastritis in horses.

Comparison between administration of *L. plantarum* 299v
and *L. plantarum* JI:1

During a short period the ten horses, who were included in the first test described above, were given fermented oatmeal gruel produced with *L. plantarum* 299v (isolated from human) which is part of the food ProViva® instead of with *L. plantarum* JI:1 (the horse strain). This was done without the knowledge of the trainer. After only one week he had realised that something was wrong with the oatmeal gruel. He had observed that the effect of the new supply of the fermented oatmeal gruel was less than before. Among other things the temperature of Luradej did not increase to slightly more than 37°C as before in spite of the horse being fed with the fermented oatmeal gruel. This shows how important it is to use the right strain of the right lactobacillus and not only the right species of lactobacilli.

CLAIMS

1. Equine feed product, comprising one or more strains of *Lactobacillus* which have been isolated from the gastric or
5 intestinal mucosa of horse and having the ability to colonize in the equine intestines.
2. Feed product according to claim 1, characterized in being a feed which has been fermented with a strain of *Lactobacillus*
10 which has been isolated from the gastric or intestinal mucosa of horse and which has the ability to colonize in the equine intestines.
3. Feed product according to claim 1 or 2, characterized in
15 comprising the strain *Lactobacillus plantarum* JI:1 DSM 11520 or a variant thereof.
4. Feed product according to claim 1 or 2, characterized in
20 comprising the strain *Lactobacillus* species AC:3 DSM 12429 or a variant thereof.
5. Feed product according to any of claims 1-4,
characterized in that the feed is cereals, vegetables, green
25 forage or hay.
6. Feed product according to any of claims 1-5,
characterized in being an oatmeal gruel fermented with
Lactobacillus plantarum JI:1 DSM 11520 or *Lactobacillus* species
30 AC:3 DSM 12429.
7. Feed product according to any of claims 1-5,
characterized in being an ensilage.
8. *Lactobacillus plantarum* JI:1 having the deposition number
35 DSM 11520 or a variant thereof.
9. *Lactobacillus* species AC:3 having the deposition number
DSM 12429 or a variant thereof.

10. Use of one or more strains of *Lactobacillus* which have been isolated from the gastric or intestinal mucosa of horse and which have the ability to colonize in the equine intestines for the preparation of a medicament for the prophylaxis or
5 treatment of disturbances of the equine intestinal flora.

11. Use according to claim 10 of the strain *Lactobacillus plantarum* JI:1 having the deposition number DSM 11520 or a variant thereof.

10 12. Use according to claim 10 of the strain *Lactobacillus* species AC:3 DSM 12429 or a variant thereof.

13. Use of one or more strains of *Lactobacillus* which have
15 been isolated from the gastric or intestinal mucosa of horse and which have the ability to colonize in the equine intestines for the preparation of a medicament for the prophylaxis or treatment of equine gastritis.

20 14. Use of one or more strains of *Lactobacillus* which have been isolated from the gastric or intestinal mucosa of horse and which have the ability to colonize in the equine intestines for the preparation of a medicament for reducing the numbers of
25 *Bacillus* ssp. and sulphite-reducing clostridia in the equine intestines.

30

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1/2

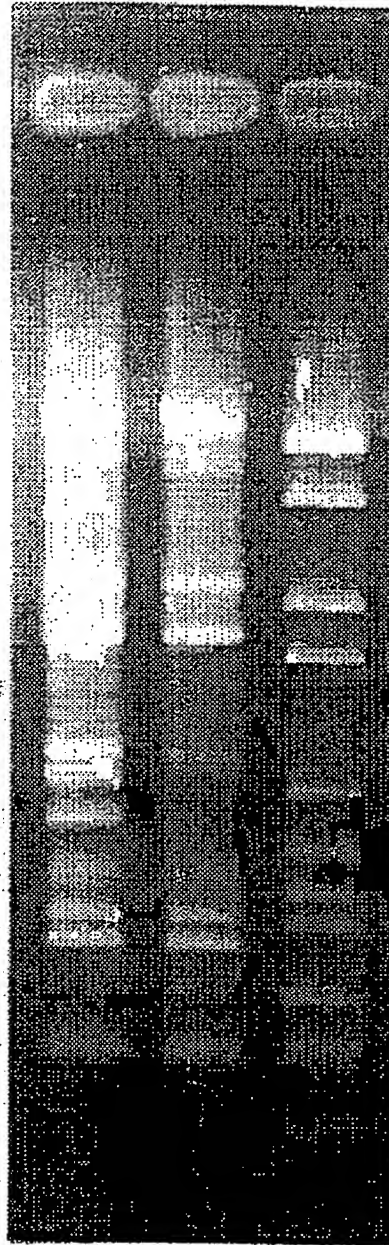


FIG. 1

SUBSTITUTE SHEET

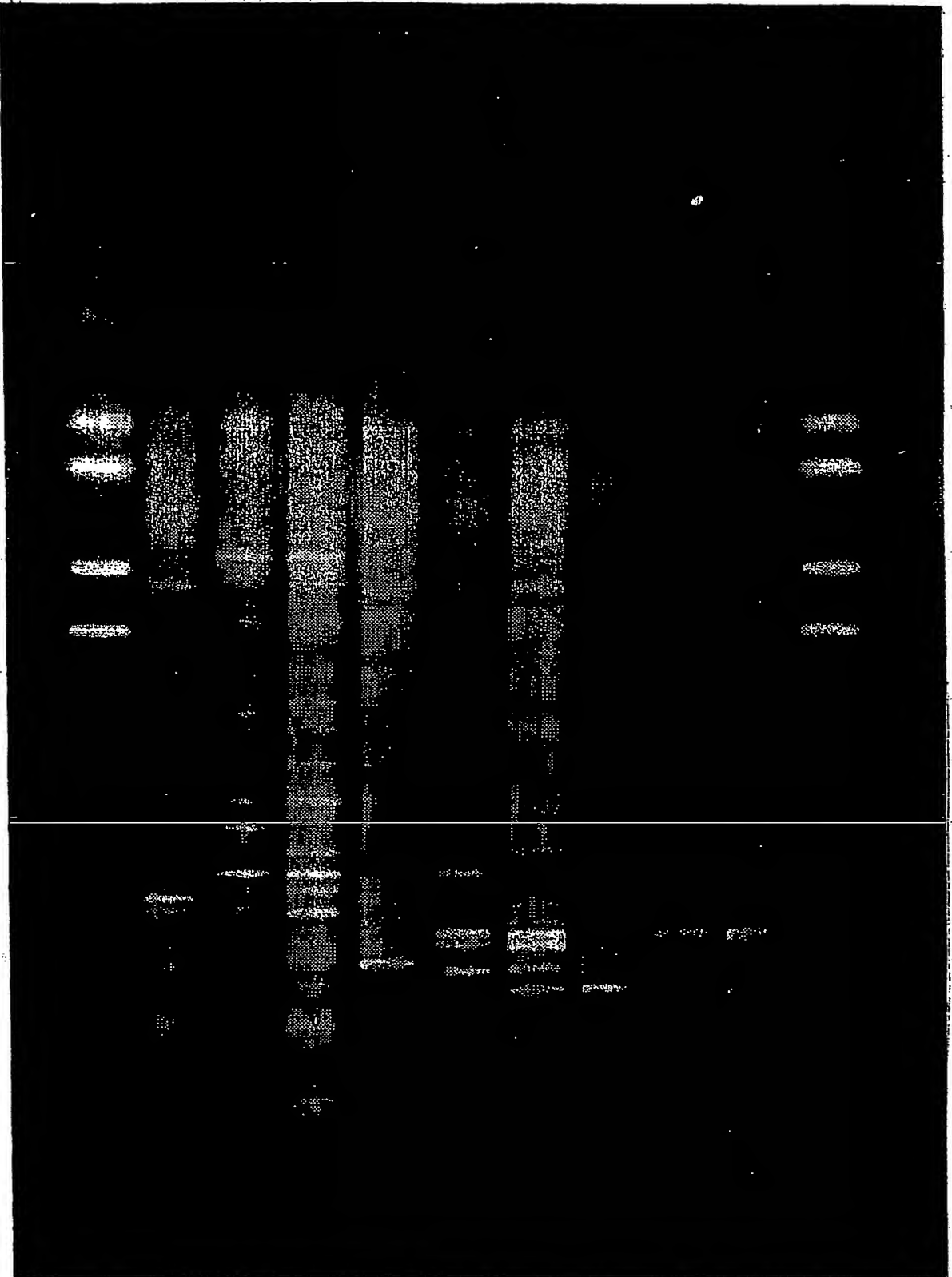


FIG. 2

SUBSTITUTE SHEET

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Probi AB
Ideon Gamma 1
Sölveg. 41

S-22370 Lund

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: JI:1	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 11520
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: <input type="checkbox"/> a scientific description <input checked="" type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 1997-04-21 (Date of the original deposit).	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) <i>V. Weis</i> Date: 1997-04-23

Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01796

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 1/20, A61K 35/74, A23K 1/16, A23K 1/18 // (C12N 1/20, C12R 1:25)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, A61K, A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5190755 A (NILS MOLIN ET AL), 2 March 1993 (02.03.93)	1,2,5,7,10, 13,14
Y	WO 9301823 A1 (PROBI AB), 4 February 1993 (04.02.93), See esp. page 19	1,2,5,7,10, 13,14

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 January 1999

Date of mailing of the international search report

20 -01- 1999

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INTERNATIONAL SEARCH REPORT
Information on patent family members

01/12/98

International application No.

PCT/SE 98/01796

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